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ROLE OF NISIN AS A BIOPRESERVATIVE IN ENHANCING THE SHELF-LIFE OF SWEET ORANGE JUICE STORED AT REFRIGERATED CONDITION (4 ±1 °C)

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ABSTRACT

A study was carried out to examine the effect of biopreservative nisin at a concentration of 0.001 mg.ml^{-1} on shelf-life of sweet orange juice packed in glass bottles, polyethylene terephtalate (PET) bottles and tin cans stored at refrigerated condition (4±1 °C). The samples were analysed for pH, total soluble solids (TSS), water activity, protein, carbohydrate, ascorbic acid, browning index and total plate count at an interval of initial, 15, 30 and 40 days of storage. It was observed that the pH, total soluble solids, protein, carbohydrates and ascorbic acid were decreased whereas, increase in water activity, juice browning index and total microbial count was noticed in all the juice samples stored at refrigerated condition. Results revealed that fresh sweet orange juice with biopreservative nisin without the addition of sugar could be usable upto 53.4 days when packed in glass bottle, 50.5 days in PET bottles and 52.3 days in tin cans. The study concludes that glass bottles are preferred for sweet orange juice along with nisin at a rate of 0.001 mg.ml^{-1} for prolonging the shelf-life of sweet orange juice at refrigerated condition

KEYWORDS: Sweet Orange Juice, Nisin and Packaging Materials

INTRODUCTION

The oranges especially the sweet orange (*Citrus sinensis (L) Osbeck)* is the fruit of the citrus species, cultivated in China for many centuries before it was introduced into Europe, most likely during the early 15th century. The fruit of sweet orange is a fleshy indehiscent berry that ranges widely in diameter from 4 to12 cm. India ranks 3rd in the production of sweet oranges after banana and mango next to China and ranks 6th in the production of citrus in the world followed by Brazil, which is the largest producer of citrus and China has the largest area under citrus production. The main citrus growing states in India are Andhra Pradesh, Maharashtra, Punjab, Haryana, Karnataka and Rajasthan. Sweet oranges are the second largest citrus fruits cultivated in the country and accounts for approximately 70% of the citrus production. The total area for sweet orange production in India during 2012-13 was 164.66 million hectare, production: 1186.41 million tonnes and productivity: 7.21 million tonnes per hectare. Maharashtra stands first in area wise production of sweet oranges in India next to Andhra Pradesh, Karnataka and Punjab (Singh and Naqvi, 2001).

Sweet oranges are rich source of vitamin A, C and potassium and supplies around 116.2% of daily value of vitamin C. It contains moisture of 86.0 g.100 g^{-1} followed by carbohydrates 12.0-12.69 g.100 g^{-1}, calcium 40-43 g.100 g^{-1}, protein 0.8-1.4 g.100 g^{-1}, fiber 0.8 g.100 g^{-1} andfat 0.2-0.4 g.100 g^{-1}. Sweet orange juice has pH 3.5, total soluble solids 10 °Brix, acidity 0.4%, moisture content 88.4%, protein 0.6%, fat 0.05%, carbohydrates 10.5%, fiber 0.12% and ash 0.3%. Sweet oranges are not available round the year so should be processed in the form of juice; concentrate, squash, etc., to minimize the post harvest losses due to spoilage (Syed *et al.*, 2012).

The beneficial effects of citrus fruit consumption on human health have long been known because of its anti-oxidant and anti-radical properties (Betoret *et al*., 2009). Citrus fruit juices are rich source of flavonoids which have important health-related properties, such as anti-microbial, anti-carcinogenic, anti-aggregative and are known to protect against cardiovascular diseases. Narirutin, hesperidin and didymin belonging to the flavanone glycosides group are the most abundant flavonoids in mandarin orange juice which have antioxidant activity and appear to influence the lipid metabolism.

Spoilage of sweet orange juice is primarily due to the proliferation of its natural acid tolerant and osmophilic microflora composed of yeasts responsible for the fermented taste accompanying carbon-dioxide production, moulds which contribute to the deterioration of juice by their surface growth and lactic acid bacteria which can grow in acidic conditions to produce a buttermilk off-flavour. In addition, pathogenic bacteria can also proliferate in fresh untreated juice during fruit picking or juice processing, representing great risk of food-borne infection with significant economic repercussions (Krinsky and Johnson, 2005).

Now a days preservation of fruit juices has become the business activity of great significance and countries with abundant fruit resources, having short harvest season are emphasizing more for established storage to maintain quality of fruits, increase shelf-life and preserve fruit juices foroff-season use (Tasnim *et al*., 2010). Fruit juices are thermally pasteurized at 63-65 °C for relatively long time and at 90-95 °C for 15 to 30 s, which is based on 5-log reduction of the most resistant microorganisms of public health significance.

Preservation of fruit juices with chemicals is mainly adopted to prevent microbial spoilage during storage, both in the retail stores and consumer homes. Nisin is an allowed preservative in a range of food products in more than 55 countries. Bacteriocin nisin, produced by *lactobacillus lactis* sub sp *lactis*, exhibits anti-microbial activity against a broad range of Gram-negative and Gram-positive bacteria including *A.acidoterrestris*. In orange juice, spore outgrowth was shown to be inhibited by addition of 25-50IU.ml^{-1}of nisin (Yamazaki *et al.*, 2000).

In order to facilitate preservation and distribution, it is technological practice to pack juices in metal cans, glass bottles or plastic containers. As for orange juice bottling, polyethylene terephtalate (PET) has been proposed as a packaging material because of its excellent barrier properties, clearness, UV resistance and good oxygen barrier properties. However, packaging alone cannot preserve the quality of juice and in addition. Therefore, juices are treated with chemical preservatives along with suitable storage temperature of 4 to 25 °C plays an important role in enhancing the shelf-life of sweet orange juice (Zerdin *et al.*, 2003).

MATERIALS AND METHODS

Materials

Sweet oranges (*Citrus sinensis (L) Osbeck)* required for the experiment were procured from local market of Raichur. Fruits were washed in tap water to remove outer dirt and extraction of juice was done by power operated screw press juice extractor followed by straining, pasteurization at 80 $^{\circ}$C for 15 min, followed by cooling, addition of nisin, filling in glass bottles, PET bottles and tin cans and stored at refrigerated condition (4±1 °C).

Preparation of Nisin Solution

Nisin solution of 1% (w/v) in 0.02N HCl was prepared by centrifuging at 2000 rpm for 20 min and the supernatant was passed through a 0.22 μm pore size filter (Millex, Millipr corp., Bedford MA, USA) to give a stock solution of 10,000 IU.ml^{-1}. The solution was stored at 4 °C for further usage (Walker *et al*., 2008).

Methods of Determination

pH

The pH of sweet orange juice was measured by using digital pH meter (make: Systronics; model: 361). Accurately 5ml of juice sample was weighed and was placed in a beaker then the electrode of the pH meter was dipped in the juice sample under test. The enter key was pressed to show the pH value and temperature of sample simultaneously (Fustier *et al*., 2011).

Total Soluble Solids

The total soluble solids (TSS) were determined according to the method described by Timmermans *et al.*, 2009, using Atago digital handheld refractometer. An appropriate quantity of sweet orange juice sample was placed on the prism of the refractometer with the help of a glass rod and pressed the start button to get the readings. For each sample, the instrument was calibrated by using distilled water. The reading appeared on the screen was directly recorded as total soluble solids (°Brix).

Water Activity

The water activity of sweet orange juice was measured by Rotronic hygrolab 3 water activity analyser. Two ml of sample under test was kept in sample cup provided with water activity meter. The sensor was placed on the sample cup by firmly closing in such a way that the air should not enter into the sample cup. The reading was directly displayed on the water activity meter and was taken as water activity of the sweet orange juice (Fustier *et al.*, 2011).

Soluble Protein

The soluble protein in sweet orange juice was determined by Lowry's metho (Rai *et al.*, 2007). Half ml extract was mixed with 10 ml of distilled water in centrifuge tube. After vortex for 2 m, the tube was centrifuged for 10 min at 2700 rpm. Supernatant was used for analysis. A volume of 0.1 ml supernatant was taken out in a test tube and after making the volume 1ml with distilled water. Three ml of reagent C were added which was made by mixing 50 ml of reagent A(2% sodium carbonate in 0.1 N sodium hydroxide) and 1 ml of reagent B (0.5% copper sulphate,1% potassium sodium tartarate).

After adding 0.2 ml of FCR reagent, tube was inverted for 30 min at room condition. Bovine albumin serum (V) was added as standard in a range of 12.50 to 100 µg.ml^{-1}. All the samples and standards were prepared in triplicate and absorbance was measured at 600 nm against a blank having all the reagents except the sample. Total protein was calculated from linear regression equation obtained from the standard curve.

Carbohydrates

The total carbohydrate content of sweet orange juice was determined using phenol sulphuric acid method (AOAC, 1999). The reagents used were 4% phenol and 96% sulphuric acid. One ml of sweet orange juice was hydrolyzed in water

bath at 60 °C for 3 hours with 2.5 N-50 ml hydrochloric acid and then cooled to room condition. The sample was neutralized with solid sodium carbonate until effervescence ceased, volume make up of sample was made upto 100 ml and centrifuged at 9000 rpm at 4 °C for 10 min to collect the supernatant and transferred into 10 ml test tube and 1 ml of phenol was added followed by 5 ml of sulphuric acid then the test tube was placed in water bath at 25-30 °C for 20 min and after cooling the absorbance was measured at 490 nm in spectrophotometer (make: Systronics; model: PC based double beam spectrophotometer 2202). To calculate the concentration of sugar present in the sample, a graph of absorbance versus concentration of sugar was plotted along with a standard curve generated from the analysis of dextrose.

Ascorbic Acid

Vitamin C (ascorbic acid) was described by titration method as described by Mazumdar and Majumder (2003). Ten grams, of sample was mixed with distilled water for 10 minutes and filtered through Whattman filter paper #4. The 10 ml sample was taken in 250 ml conical flask and 15 ml 21% oxalic acid was added. The sample was titrated with 2% dichlorophenol indophenols till pink colour appeared. The results were calculated using the following formula and expressed in mg.100 g^{-1} fresh weight.

$$\text{Ascorbic acid (mg/100ml)} = \frac{\text{Titre} \times \text{Dye factor} \times \text{volume}}{\text{Volume of filtration taken} \times \text{volume of sample}}$$

Browning Index

The juice browning index was estimated as follows: orange juice (10 ml) was centrifuged for 10 min at 7800 rpm at 4 °C. Five ml of ethanol (95%) was added to 5ml of the supernatant and centrifugation was repeated. The absorbance of the supernatant was read at 420 nm using a UV-visible spectrophotometer with a cell path length of 1 cm by keeping 95% ethanol as a standard (Fustier *et al.*, 2011).

Total Plate Count

On each sampling day, 10 ml portion of sweet orange juice was aseptically weighed into 90 ml of sterile water and blended for 15 min at room temperature. From this, 1 ml of the sweet orange juice sample was accurately pipette, using a micropipette into test tubes containing 9 ml of sterile distilled water (10^{-1}) and serially diluted until 10^{-6} dilution was reached. One ml aliquot from 10^{-3}, 10^{-4} and 10^{-5} dilutions were transferred to the sterile petriplates for the enumeration of fungi, yeast and bacteria, respectively. Plates were duplicated for each dilution. Approximately 15-20 ml of molten and cooled media, plate count agar at 45 °C) for the respective organisms were added to the petriplates and the plates were rotated clockwise and anti-clockwise directions on the flat surface to have a uniform distribution of colonies. After the solidification of agar, the plates were inverted and incubated at room temperature for 2-5 days (bacteria 2 days, yeast and fungi 5 days). Total plate counts were determined on plate count agar pour plates and enumerated after an incubation period of 48-72 h at 30 °C (Rahman *et al*., 2011). The colonies were counted after the incubation period and the number of cfu per ml of sample were calculated by applying the following formula:

$$\text{No of cfu/ml of the sample} = \frac{\text{Mean number of cfu} \times \text{Dilution factor}}{\text{Volume of the sample}}$$

Where,

Dilution factor is the reciprocal of the dilution (e.g. $10^{-3} = 10^{3}$)

Shelf-Life Assessment of Sweet Orange Juice Based on Ascorbic Acid Content

The shelf-life studies of sweet orange juice was calculated by taking into account the ascorbic acid concentration changes upon storage and by taking the prescribed ascorbic acid value of 40 $mg.ml^{-1}$ which was considered as the limit value to predict the shelf-life of citrus juices (Ribeiro *et al.*, 2009). The ascorbic acid concentration changes would follows the pseudo-first order kinetics rate constant and the required intercept and goodness of fit values were obtained from MATLAB by plotting the values of ascorbic acid on Y-axis and storage period on X-axis and shelf-life of the sweet orange juice was calculated by using the formula

$$SL = \frac{ln(AA) - Intercept}{Rate\ constant(K)}$$

Where AA is the ascorbic acid concentration corresponding to the acceptability limit

Statistical Analysis

Statistical analysis was carried out to study the effect of different parameters on all the dependent variables by Completely Randomized Design (CRD) using the statistical software AGRES. Analysis of variance (ANOVA) was conducted to determine whether significant effect of packaging and preservation methods used to enhance the shelf-life of sweet orange juice.

RESULTS AND DISCUSSIONS

pH

The effect of biopreservative nisin and packaging materials on pH of sweet orange juice was analysed and presented in Table (1). It was clear that as the storage period proceeds, the pH decreases in all the samples, whereas less decrease in pH was observed in glass bottles, followed by tin cans and PET bottles. This is due to acidity stabilizing effect of nisin in combination with lower temperature that prevents the formation of free acid radicals and hydrolysis pectin by creating unfavourable condition for enzymes and microorganisms to grow and multiply (Cecilia and Maia. 2002).

Total Soluble Solids (TSS)

The effect of biopreservative nisin and packaging materials on total soluble solids of sweet orange juice, stored at refrigerated condition (4±1 °C) was analysed and presented in Table (2). It was noted that as the storage period proceeds, the decrease in total soluble solids of sweet orange juice takesplace in all the samples but less in juice sample preserved with nisin, filled in glass bottles, followed by tin cans and PET bottles, stored at refrigerated condition. This decrease in TSS might be due to utilization of sugars by fermenting organisms leading to degradation of sugars and moreover, decrease could also be attributed to the precipitation of tannins and colloidal particles in the juice costa *et al.* (2003).

Table 1: Effect of Biopreservative Nisin and Packaging Materials on pH of Sweet Orange Juice, Stored at Refrigerated Condition (4±1 °C)

pH				
Treatments	**Day 1**	**Day 15**	**Day 30**	**Day 40**
T1	3.78	3.46	3.34	3.27
T2	3.57	3.38	3.26	3.20
T3	3.71	3.40	3.28	3.25
T4	4.21	4.19	4.06	3.90
T5	4.08	4.02	3.95	3.83
T6	4.15	4.10	4.04	3.85
S.Em±	0.081	0.075	0.082	0.079
CD @ (1%)	0.350	0.325	0.355	0.340
CV	3.585	3.468	3.907	3.848
Factor	S	S	S	S

pH of Fresh Sweet Orange Juice- 4.38

NS: Non significant, S: Significant

Table 2: Effect of Biopreservative Nisin and Packaging Materials on Total Soluble Solids (TSS) of Sweet Orange Juice, Stored at Refrigerated Condition (4±1 °C)

Total Soluble Solids (°Brix)				
Treatments	**Day 1**	**Day 15**	**Day 30**	**Day 40**
T1	11.08	7.55	6.72	5.58
T2	11.05	7.46	6.68	5.50
T3	11.07	7.48	6.69	5.52
T4	11.22	10.20	8.21	6.78
T5	11.17	10.08	8.13	6.71
T6	11.19	10.15	8.16	6.74
S.Em±	0.172	0.144	0.107	0.108
CD @ (1%)	0.742	0.623	0.464	0.466
CV	2.672	2.836	2.502	3.044
Factor	NS	S	S	S

Total Soluble Solids Content in Fresh Sweet Orange Juice- 10.9 °Brix

NS: Non significant, S: Significant

Water Activity

The effect of biopreservative nisin and packaging materials on water activity of sweet orange juice was analysed and presented in Table (3). It was noted that that as the storage period proceeds, the increase in water activity was observed in all the samples. This might be due to availability of free water for the growth and multiplication of microbes in sweet orange juice with respect to storage period even at refrigerated condition among packaging materials glass bottle was found to be best in minimizing the water activity, followed by tin cans and PET bottles, along with preservatives was found to be best in controlling water activity (Chumillas *et al.*, 2007).

Soluble Protein

The effect of biopreservative nisin and packaging materials on soluble protein of sweet orange juice, stored at refrigerated condition (4±1 °) was analysed presented in Table (4). It was clear that as the storage period proceeds soluble

protein content decreases and due to lower temperature protein degradation was found to be less. The loss of soluble protein might be due to the denaturation of protein when subjected to pasteurization temperature of 80 °C for 20 s, as it is heat sensitive and also breakdown of protein by microoraganisms as storage period increases which is found to be less when stored at lower temperature Idah *et al.* (2010).

Table 3: Effect of Biopreservative Nisin and Packaging Materials on Water Activity of Sweet Orange Juice, Stored At Refrigerated Condition (4±1 °C)

Water activity				
Treatments	**Day1**	**Day 15**	**Day 30**	**Day 40**
T1	0.881	0.893	0.905	0.917
T2	0.889	0.901	0.913	0.925
T3	0.883	0.895	0.907	0.919
T4	0.849	0.858	0.865	0.880
T5	0.855	0.864	0.877	0.886
T6	0.851	0.86	0.872	0.884
S.Em±	0.003	0.002	0.002	0.002
CD @ (1%)	0.011	0.009	0.009	0.007
CV	0.526	0.433	0.425	0.292
Factor	S	S	S	S

Water Activity of Fresh Sweet Orange Juice- 0.882

NS: Non significant, S: Significant

Table 4: Effect of Biopreservative Nisin and Packaging Materials on Soluble Protein Content of Sweet Orange Juice, Stored at Refrigerated Condition (4±1 °C)

Soluble protein (%)				
Treatments	**Day 1**	**Day 15**	**Day 30**	**Day 40**
T1	0.36	0.34	0.29	0.22
T2	0.32	0.28	0.25	0.19
T3	0.34	0.32	0.26	0.20
T4	0.44	0.42	0.39	0.33
T5	0.42	0.39	0.36	0.27
T6	0.43	0.40	0.37	0.31
S.Em±	0.016	0.015	0.014	0.014
CD @ (1%)	0.069	0.066	0.061	0.061
CV	7.209	7.334	7.684	9.713
Factor	**S**	**S**	**S**	**S**

Soluble Protein in Fresh Sweet Orange Juice- 0.54%

NS: Non significant, S: Significant

Carbohydrates

The effect of biopreservative nisin and packaging materials on carbohydrates of sweet orange juice, stored at refrigerated condition (4±1 °C) was analysed and presented in Figure. (1). It was observed that as the storage period proceeds, decrease in carbohydrates takesplace in all the samples. However, carbohydrates degradation was found to be less in juice sample preserved with nisin when compared to controlled samples stored at refrigerated condition which provides unfavourable condition for the growth and multiplication of microorganisms by converting carbohydrates into

fermentable sugar, which was found to be one of the most suitable hurdle technology in inhibiting the microbial load and maintaining the overall quality of the juice (Meyer, 2004).

Ascorbic Acid

The effect of biopreservative nisin and packaging materials on ascorbic acid content of sweet orange juice, stored at refrigerated condition (4±1 °C) was analysed and presented in Figure (2). It was observed that as the storage period proceeds, the decrease in ascorbic acid takesplace in all the samples but whereas in juice sample preserved with nisin filled in different packaging materials, stored at refrigerated condition was found to be less. This might be due to non-volatility, water solubility and broad antimicrobial activity of bacteriocin nisin in retaining the ascorbic acid content of fruit beverage at lower temperature (Walker, 2008).

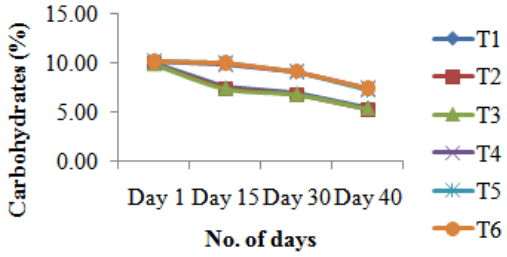

Figure 1: Effect of Biopreservative Nisin and Packaging Materials on Carbohydrates (%) of Sweet Orange Juice Stored at Refrigerated Condition (4±1 °C)

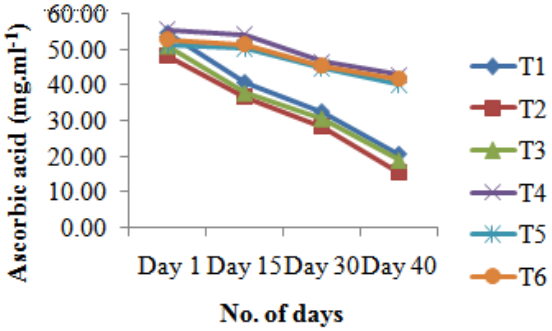

Figure 2: Effect of Biopreservative Nisin and Packaging Materials on Ascorbic Acid ($Mg.Ml^{-1}$) Content of Sweet Orange Juice Stored at Refrigerated Condition (4±1 °C)

Browning Index

The effect of biopreservative nisin and packaging materials on browning index of sweet orange juice, stored at refrigerated condition (4±1 °C) was analysed and presented in Figure (3). It was observed as the storage period proceeds, browning index of sweet orange juice also increases but less when compared to juice preserved with nisin. This might be due to increase in browning index upon storage that involves reaction between ∝-amino groups and reducing sugars to yield brown pigments at high temperature treatments which can be controlled by addition of nisin along with low temperature and suitable packaging material (Fustier *et al.*, 2011).

Total Plate Count

The effect of biopreservative nisin and packaging materials on total plate count of sweet orange juice, stored at refrigerated condition (4±1 °C) was analysed. It is observed from the Figure. (4) as the storage period proceeds, the microbial count also increases in all the samples but whereas in juice samples preserved with nisin, the total plate count

was found to be less. This is due to the effectiveness of bacteriocin nisin in combination with packaging materials plays an important role in inhibiting the growth of vegetative spores and microbial spores at lower temperature (Komitopoulou *et al.*, 1999).

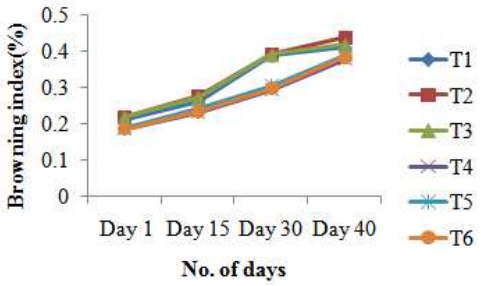

Figure 3: Effect of Biopreservative Nisin and Packaging Materials on Browning Index (%) of Sweet Orange Juice Stored At Refrigerated Condition (4±1 °C)

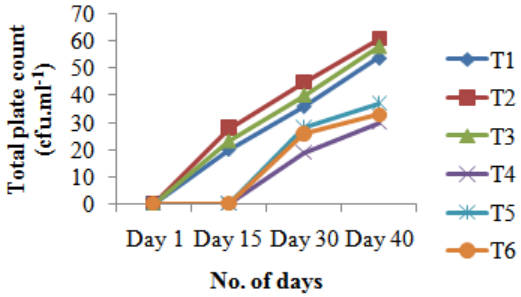

Figure 4: Effect of Biopreservative Nisin and Packaging Materials on Total Plate Count (Cfu.Ml^{-1}) of Sweet Orange Juice Stored at Refrigerated Condition (4±1 °C)

Shelf-life assessment of sweet orange juice based on ascorbic acid content

The effect of biopreservative nisin and packaging materials on shelf-life of sweet orange juice, stored at refrigerated condition (4±1 °C) based on ascorbic acid content limit was analysed and presented in Table (5). It is observed that the shelf-life of sweet orange juice can be extended upto 25.5, 20 and 21 days, followed by 53.4, 50.5 and 52.3 days along with preservative when filled in glass bottles, PET bottles and tin cans, stored at refrigerated condition. This might be due to effectiveness of biopreservative nisin was sufficient enough enough to stop the yeast, moulds and microbial activity at lower temperature at 4 $^{\circ}$C in extending the shelf-life of juice Esteban and palop (2011).

Table 5: Effect of Different Biopreservative and Packaging Materials on Shelf-Life Prediction of Sweet Orange Juice, Based on Ascorbic Acid Limit Stored at Refrigerated Condition (4±1 °C)

Treatments	DAYS	K- Value	C-Value	SSE	R^2	Adjusted-R^2	RMSE
T1	14.4	-0.0241	4.037	0.0147	-0.9732	0.9597	0.0857
T2	9.8	-0.0264	3.948	0.0684	0.9039	0.8559	0.185
T3	11.8	-0.0229	3.96	0.0362	0.9302	0.8954	0.1346
T4	53.4	-0.0066	4.04	0.0035	0.918	0.8769	0.0421
T5	50.5	-0.0051	3.956	0.0105	0.6937	0.5406	0.0727
T6	52.3	-0.0059	3.987	0.0028	0.9197	0.8795	0.0038
S.Em±	1.52						
CD @ (1%)	6.01						
CV	5.45						
Factor	S						

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CONCLUSIONS

It was observed that the sweet orange juice preserved with nisin, filled in various packaging materials maintained overall physico-chemical properties, when compared to that of the controlled samples stored at refrigerated condition of 4±1 °C. However, sweet orange juice filled in glass bottle along with the biopreservative showed the maximum shelf-life of 53.4 days with a least microbial count compared to PET bottles and tin cans, which showed shelf-life of 50.5 and 52.3 days at refrigerated condition. This shows that glass acts as a good packaging material in enhancing the shelf-life of sweet orange juice followed by tin cans and PET bottles

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